Effects of SiO₂ nanoparticles on phospholipid membrane integrity and fluidity

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HIGHLIGHTS

- SiO₂ NPs tend to disrupt oppositely-charged membranes.
- Particle sedimentation aggravates membrane disruption.
- SiO₂ NPs induce membrane gelation.

GRAPHICAL ABSTRACT

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ABSTRACT

Silicon nanoparticles (NPs) are widely used nanomaterials and reported to have pathogenicity. Effects of five different SiO₂ NPs on the membrane integrity and fluidity were studied using giant unilamellar vesicles (GUVs) as model cell membranes. GUVs were made from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) by gentle hydration method, and adjusted to be positively- or negatively-charged by adding charged lipids into vesicles. SiO₂ NPs caused more serious damage to oppositely-charged membrane because electrostatic attraction favored the hydrogen bonding to the phospholipids. Increase in NP exposure dose/time and NP sedimentation process aggravated the membrane damage. The membrane phases were evaluated applying the fluorescent probe Laurdan and the calculated generalized polarization (GP) values. Anionic SiO₂ NPs increased the GP value and induced membrane gelation. Cationic SiO₂ NPs did not change the phase of positively-charged GUV and pure DOPC vesicles, but induced the gelation of negatively-charged GUV. Break of membrane integrity and change in membrane phase are possible mechanisms of cytotoxicity because cellular physiological activities require a separated intracellular environment and a fluid membrane phase to support proteins and regulate molecular transport.

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1. Introduction

The fast-developing nanotechnology provides a board usage of nanomaterials in both industrial products and public daily life [1,2]. Silicon dioxide distributes widely in environment as the chief component of quartz and glass, and its NPs have been employed in the

semiconductors, batteries, catalysis, inorganic paints, drug delivery and imaging [3,4]. Their potential exposure routes include inhalation, ingestion, skin uptake, and injection. After that, they may pass through the skins and mucous membranes, enter bloodstream, and transport in the body [5,6]. SiO₂ NPs may lead to silicosis at low intensity, induce risk of pulmonary fibrosis, and lung cancer in long-term exposures, and are listed as highly toxic dust particles [7,8]. SiO₂ NPs also can induce hemolysis of human red blood cells [9]. Therefore, the toxicities of SiO₂ NPs became a public concern

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and were studied in recent years [10–13], but the etiology of their biological toxicity remains controversial.

Direct contact of NPs with cell membrane may carry the risk of membrane rupture and cytotoxic effects [14]. Cell membrane is a phospholipid bilayer with the main roles of separating intracellular and extracellular environments, and providing an anisotropic fluid phase to support proteins and to regulate molecular transport. The membrane integrity and fluidity are essential and important to cell physiological activities, which may be affected by NP attachments [12,15]. NP-induced plasma membrane disruption has been suggested for a wide range of NPs through lactate dehydrogenase (LDH) assay [16]. Membrane phase transition was observed when phospholipid membranes interact with charged polystyrene NPs [15]. The biological effects of NPs are well known to closely relate to their properties. Positively-charged NPs have been reported to interact strongly with cell membrane and induce cytotoxicity [17–19], which show the role of electrostatic interaction. However, in a study on gold NPs both the positively- and negatively-charged NPs induce membrane leakage [20], indicating that electrostatic interaction is not the only factor. Pathogenicity of crystalline silica is suggested to be largely attributed to the presence of silanol groups at the silica surface in many previous researches [8,9], because silanol hydroxyl groups can form strong hydrogen-bond with organic phosphate groups [8]. Silanol bonding and other physisorption possibly form between SiO_2 NPs and the phosphate headgroups of cell membrane [21]. Considering the previous reports on the SiO_2 NP toxicities and the NP-membrane interactions, we hypothesize that SiO_2 NP exposure may induce membrane disruption and phase change. The damages to cell membrane possibly are important mechanisms of cytotoxicity. Therefore, this research aims to explore the changes in cell membrane integrity and fluidity caused by SiO_2 NPs.

However, the ingredient of natural cell membranes and uncertainty in the incubating environment make the difficulties to evaluate the NP-induced direct membrane changes and to determine the interaction mechanisms. Giant unilamellar vesicles (GUVs) are ideal membrane models to reduce complexity and uncertain factors, and to eliminate the effects of cell metabolism and growth [22], moreover are more targeted for mechanism exploration by systematically varying membrane composition. GUVs are similar to real cell in size and external curvature, and visible under the optical microscopy [6,21], which are more close to cell membrane than other model membranes and have the advantages of both direct morphological observation and phase state quantification. Therefore, the results obtained using GUVs may provide the mechanism explanation to the cytotoxicity of NPs.

SiO_2 NPs are often made to stable colloids by adding stabilizer, or are specially coated to achieve desirable functions, or are produced to porous particles to obtain larger surface area and utilization efficiency. Those treatments may change their properties and result in their different toxicities. Therefore, five SiO_2 NPs include colloids and powders, positively and negatively-charged NPs, porous and non-porous particles were selected in our experimental design to better understand the effects of commonly-used SiO_2 NPs on cell membrane. The changes in membrane integrity and phase were explored using GUV model membrane through microscopic imaging and fluorescence analysis.

2. Materials and methods

2.1. Materials

The colloidal silica NPs (LUDOX AS/CL/LS colloidal silica) were purchased from Sigma–Aldrich, and their surface configurations were presented in Supplementary data Fig. S1. SiO_2 NP powders (DP1 and DS1) are purchased from Zhejiang Hongsheng

Material Technology Co., China. DP1 and DS1 are porous and non-porous NPs, respectively. The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DPPG), 1,2-dipalmitoyl-3-trimethylammonium-propane (chloride salt) (16:0 TAP), and fluorescent lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (RhB-PE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Fluorescent probe 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) was bought from Molecular Probes.

2.2. The measurement of NP properties

The zeta potentials (pH 3–9) and the hydrodynamic diameters ($\text{pH} \approx 6.5$) of NPs were measured by a Malvern zetasizer in 0.1 M glucose. The single particle diameters were measured under TEM, and the BET surface areas of NPs were provided by the manufacturer.

2.3. GUV making

GUVs were made using the gentle hydration method [23]. DOPC, DPPG and 16:0 TAP were dissolved in chloroform:methanol (2:1 by volume) to make lipid solutions of 18 mg/mL, 2 mg/mL and 2 mg/mL, respectively. 50 μL of DOPC was mixed with 50 μL of DPPG or 16:0 TAP to produce negatively-charged (GUV^-) or positively-charged vesicles (GUV^+). The fluorescent vesicles were made by adding 0.1% (w/w) of RhB-PE to the lipid mixture. The Laurdan-labeled vesicles were made by adding 0.4% of Laurdan probes into the lipids. Then the 100 μL of lipid solution was dried under nitrogen blow on a rotary evaporator to form a thin phospholipid film inside a glass tube. The tube with lipid film was kept in a desiccator in vacuo for more than 6 h to remove organic solution residues. After that, the tube was filled with 0.1 M sucrose (about 4.5 mL) and incubated at 40 °C for 24 h to form GUVs. The GUV suspension was diluted (v:v = 1:15) in 0.1 M glucose before microscopic observation.

2.4. GUV observation

Bright field (BF) observations were conducted using an inverted microscope with 40 $\times$ objective lenses (DSY5000, UOP Photoelectric Technology). The internal sucrose and the external glucose made difference in the refractive indices of the inner and outer vesicle media, and made vesicles visible under a BF microscopy. The fluorescence of RhB-PE labeled vesicles was imaged by Carl Zeiss LSM 700 confocal microscopy.

2.5. Exposure experiments

After 30-min sonication, 280 μL of NP suspension was put into a glass bottom (30 mm diameter, 0.15 mm thickness) petri dish, and then 20 μL of vesicle suspension was added to mix with NPs in glucose. The effects of NP dose were studied by adjusting NP suspension from 0 to 100 mg/L. The morphologies of vesicles after NP exposure were recorded under microscopy at 5 min and 24 h, respectively. The remaining amount of GUVs after NP exposure was counted according to the method in Supplementary data. To study the effects of exposure time, the GUVs were dyed by RhB-PE and the morphology changing was recorded under confocal microscopy (Zeiss, LSM 700).

2.6. Fluorescence spectrum measurements

Laurdan-labeled GUVs ~ 25 mg/L were exposed to 50 mg/L SiO_2 NPs to study the membrane fluidity. Fluorescence scans were

Table 1Physiochemical properties of studied SiO₂ NPs. The particle hydrodynamic diameter and zeta potential were measured in 0.1 M glucose.

Particles	Stabilizing counterion	Single particle diameter (nm)	Hydrodynamic diameter (nm)	Surface area* (m ² /g)	Zeta potential (pH 6.5, mV)
AS	Ammonium	40–70	46.1	~230	–25.59
CL	Chloride (alumina coated)	~20	47.7	~230	6.19
LS	Sodium	~20	61.5	~215	–32.64
DP1	–	20–50*	198.8	700 ± 30	–32.58
DS1	–	20–50*	234.5	200 ± 20	–5.48

*Provided by manufacturer.

conducted by a spectrophotofluorometer (Hitachi F-4500). The fluorescent dye Laurdan was excited at 345 nm, and its emission spectrum was collected at 400–550 nm. The emission intensity is normalized after dividing the maximum intensity and an excitation-emission plot was conducted at final. The Laurdan emission intensity was selected at 440 and 490 nm to calculate the generalized polarization (GP) by the following equation [24]:

$$GP = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})}$$

GP value ranges from –1 to +1, reflects the phospholipid membrane phase. GP values >0.55 and <–0.05 are gel and fluid phase, respectively. The intermediate between gel and fluid phases is divided into liquid-disordered (–0.05 < GP < 0.25) and liquid-ordered (0.25 < GP < 0.55) phases [25].

3. Results and discussion

3.1. Properties of NPs

The physiochemical properties of the five SiO₂ NPs are listed in Table 1. The single particle diameter was measured under TEM (Fig. S2) and the hydrodynamic diameter was measured by dynamic light scattering. The particle hydrodynamic diameters are usually larger than the single particle diameter due to the particle aggregation. The SiO₂ powders (DP1/DS1) aggregate to larger size than the colloids (AS/CL/LS). The zeta potentials of studied SiO₂ NPs from pH 3 to 9 are presented in Fig. 1. The particle surface charge decreases when pH value increases for all the NPs. In the pH region (pH ≈ 6.5) of NP exposure experiment, CL SiO₂ NPs are positively-charged, others are negatively-charged.

Fig. 1. Zeta potentials of AS/CL/LS/DP1/DS1 SiO₂ NPs in pH 3–9. The zeta potentials of GUV⁺ and GUV[–] are measured at pH 6.5 in 0.1 M glucose.

3.2. Influence of surface charge, exposure dose and time

On the BF images (Figs. 2 and 3), the colloidal SiO₂ NPs cause serious rupture of the oppositely-charged GUVs, the counted GUV amount after NP exposure are presented in Supplementary data Table S1. Negatively-charged AS and LS SiO₂ NPs break almost all the GUV⁺ at 50 and 100 mg/L, but the majority of GUV[–] remains intact (Table S1). Positively-charged CL SiO₂ NPs cause the reverse result (Figs. 2 and 3, Table S1). SiO₂ NP powders (DP1 and DS1) show obvious effects on the morphology of both GUV⁺ and GUV[–] (Figs. 2 and 3), but they break more oppositely-charged GUV⁺ than the like-charged GUV[–] (Table S1). Above phenomena indicate that the electrical interaction between membrane and NPs is an important factor to cause membrane disruption. However, in previous studies membrane rupture was not observed after GUVs were exposed to oppositely-charged NPs [21,26]. Therefore it was concluded that NP-membrane interaction was not ruled by the global charge attraction, but by surface properties [21]. Considering all those observations, electrostatic force may not cause membrane disruption itself, but it attracts the NPs to membrane surface, which is a keypoint to break the membrane.

The influences of exposure dose (0–100 mg/L) are also presented in Figs. 2 and 3 and Table S1. Obviously lower concentrations of NPs have less effect on GUV amount and morphology. As the dose increases, more NPs can attach on GUVs. Not only the shape of GUVs is altered, but also the integrity of lipid membrane is broken. The amount of remaining GUVs decreases when the NP dose increases from 10 to 100 mg/L (Table S1). On the BF images, GUVs are damaged into pieces under the exposure of 50 and 100 mg/L oppositely-charged NPs, but under 10 mg/L NPs, most GUVs are not affected. These observations indicate that NPs need to reach certain amount to break the membrane.

During the exposure experiment, vesicle ruptures are not observed at 5 min (Figs. S3 and S4). Even the high dose of oppositely-charged NPs does not show obvious effects, which shows that NP interaction with GUV also need time to induce GUV rupture. RhB-PE labeled GUVs are observed under a confocal fluorescence microscopy to record the vesicle changing process (LS NPs are the example, Fig. 4). Some GUVs are disrupted to form fluorescent pieces in 120 min (upper images). The lower images present the details how a vesicle (yellow arrow marked) disappears gradually at 22–26 min. First some bright fluorescent spots appear on the membrane and grow larger, meaning that NPs attach on the GUV and gather the surrounding lipid bilayers, so the vesicle shrinks. Then more and more fluorescence substances appear inside the vesicle, indicating that phospholipid-covered NPs are engulfed into the GUV. Finally the vesicle disappears. Engulf of silica NPs into giant vesicles was reported by a cryo-TEM study [27]. Adhesions of particles onto GUVs induce the coverage of particle by lipid bilayers and accumulation of phospholipids to the particles. When the vesicles engulf particles together with their attached phospholipids, the size of GUVs shrinks because the phospholipids building the vesicle shell now become inside the vesicles.

Fig. 2. Morphology of GUV⁺ after 24 h exposure of NPs (0–100 mg/L) in 0.1 M glucose.

Lipid membranes probably attach on the NP surface due to electrostatic interaction and other intermolecular forces [2,28], which may be the reason of GUV damage. When NPs and GUVs are oppositely-charged, the electrostatic force attracts the NPs onto the membrane surface. When NPs are close enough to membrane, $R-N(CH_3)_4^+$ or $(PO_4)^-$ groups in phospholipids may form high affinity with NP surface hydroxyl groups [21]. It has been reported that a strong affinity lies between the silica particle and phosphatidylcholine [21,29]. Silanol groups (Si–OH) on the silicon dioxide surface can form strong hydrogen-bond complexes with biological molecules, especially with organic phosphate groups [8]. Hydrogen bonding between the Si–OH and the O–P groups of phospholipids was confirmed by a NMR study on the interaction of silica with liposome [30]. The strong affinity makes the membrane deform to cover more NP surface [27] and induce a driving force to tear membrane. The driving force can be neutralized through vesicle shrinking and engulfing NPs inside to reach a new balance. When the NPs attached on membrane reach certain amount and

the driving force exceeds the hydrophobic interaction among phospholipid molecules, the membrane rupture or cell leakage happens. The integrated vesicles are torn into pieces which are confirmed by fluorescent images (Fig. 4). In cells, those NP-membrane interaction processes increase levels of reactive oxygen species, and lead ultimately cell damage [8].

3.3. Comparison of SiO₂ NP colloids and powders

The DP1 and DS1 NPs are SiO₂ powders without stabilizing counterions as compared with the stable SiO₂ NP colloids. Different to silica colloids, DP1 and DS1 NPs even cause serious membrane disruption to their like-charged GUVs (Figs. 2 and 3), decrease their amount to 23.6% and 17.7% compared to the control (Table S1). According to the results of 30-min exposure, DP1/DS1 powders present faster damage to the oppositely-charged GUVs than AS/CL/LS colloids (Fig. 5 and Table S2). The likely reason is that SiO₂ NP powders are easier to aggregate and then sediment to the

Fig. 3. Morphology of GUV[−] after 24 h exposure of NPs (0–100 mg/L) in 0.1 M glucose.

bottom from the bulk solution compared with the colloids (Table 1 and Fig. S5). Sedimentation was reported that it can increase the cellular uptake of NPs because it increased the dose of NPs on the cell surface [31]. When SiO₂ NP powders form large and heavy aggregates, its sedimentation velocity is much larger than the diffusion velocity, which causes NPs to sediment quickly. GUVs also settle and approach the bottom after standing for a while, therefore sedimentation increases the amount of NPs in the interaction zone between bulk solution and phospholipid membrane, and the dose of NPs attaching on membrane. Because the GUV damage relates to the NP exposure dose, it is reasonable that powders cause more serious damage than colloids. Moreover, when the formed large NP aggregates hit on the GUVs during their sedimentation, the gravity of larger aggregates will cause pressure on the membrane and increase the interaction between NPs and membrane, which will induce vesicle deformation and help to break the membrane. In addition, hydrogen bonding between silica and phospholipids may

be disturbed by the counterions surrounding the colloids, which probably relates the slower membrane disruption.

The DP1 SiO₂ NP powders are porous particle and have much higher surface area than its nonporous counterpart DS1 (Table 1). However, there are no obviously differences between DP1 and DS1 NPs when they are exposed to GUVs. The Si–OH groups inside the pores may be not involved in the NP-membrane interactions.

3.4. Influence on membrane fluidity studied by fluorescence spectra

The fluorescent spectra of Laurdan-labeled vesicles are presented in Fig. 6. In gel-phase membrane, few water molecules present in the membrane and the emission maximum of Laurdan is at around 440 nm. In fluid-phase membrane there are more water molecules inside the membrane, therefore the energy required for the reorientation of surrounding water dipoles decreases the exited

Fig. 4. RhB-labeled GUV⁺ changes after LS SiO₂ NP exposure (50 mg/L) are recorded by confocal fluorescent microscopy. Upper images show the differences between 5-min and 120-min exposure. The lower images show the process of how a GUV (yellow arrow marked) shrinks and disappears. The white star marked vesicle does not change and is used as the reference of size. Scale bars, 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Comparison between SiO₂ NP powders (50 mg/L) and colloidal (50 mg/L) after 30-min exposure.

state energy of the Laurdan probe. Such energy decrease results in the red-shift (shift to longer wavelength) of Laurdan maximum emission to 490 nm [24]. The relationship between Laurdan emission and membrane phase is diagramed in Fig. S6. The calculated GP values are applied to represent the membrane phase accurately (Table 2). GP values are <-0.05 in GUV⁺, GUV⁻ and pure DOPC vesicles (Table 2), indicating the fluid-phase of the DOPC membrane at 20 °C [25]. The addition of charged phospholipid molecules (16:0 TAP and DPPG) to DOPC does not change the membrane phase. After NP exposure, most silicon NPs cause Laurdan to exhibit blue-shift (shift to shorter wavelength), which means membrane gelation [24,32]. According to the GP values (Table 2), they change fluid-phase membrane to the intermediate phase between fluid and gel

($-0.05 > GP > 0.55$). However, CL NPs (cyan dot line, Fig. 6) only cause spectrum blue-shift and membrane gelation of GUV⁻, but do not change the phase of GUV⁺ and pure DOPC vesicles (Table 2 and Fig. 6). Moreover, the GP values of 24-h interaction are higher than 1-h interaction in Table 2, indicating that exposure time increase the membrane gelation.

The membrane gelation can be explained by the interaction between NPs and the P⁻–N⁺ dipoles in phospholipid headgroups. In fluid-phase membrane, P⁻–N⁺ dipole is nearly parallel to the local bilayer plane with an average angel of 0–3° [33]. Anionic NPs interact preferentially with N⁺ terminus and raise the tilt angle of P⁻–N⁺ dipole, increase lipid density and induce membrane gelation [15]. Such explanation focuses the effect of electrostatic

Fig. 6. Fluorescence spectra of Laurdan-labeled vesicles shift after interaction with AS/CL/LS/DS1/DP1 SiO₂ NPs. Most NPs move the maximum fluorescence emission to shorter wavelength. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

forces and in our study fits the performance of anionic silicon NPs (AS/LS/DP1/DS1) very well. Conversely, binding of cationic NPs to phospholipid zwitterionic headgroup is not encouraged electrostatically and geometrically because headgroup terminates with positive charge [33], which can explain that cationic CL NPs do

not change the membrane phases of GUV⁺ or pure DOPC vesicles. However, CL NPs induce gelation of GUV⁻, which suggests other mechanisms to change membrane phase. When the membrane is strongly negatively-charged, long-range electrostatic force attracts cationic CL NPs to membrane surface. Then short-range hydrogen bonds cause the binding of NPs to (PO₄)⁻ group and provide strong connection between membranes and NPs. Therefore, phospholipid molecules are fixed, and the mobility of phosphate moiety and acyl chains are decreased [30], which decreases the water penetration into lipid bilayer and changes the dipolar relaxation of Laurdan probe, and thus shifts its emission spectra [24].

Hydrogen bonding causes the fixing of phospholipid molecules on NP surface, which is an important factor to induce membrane gelation besides the tilt angle change of P⁻—N⁺ dipole caused by electrostatic forces. For anionic NPs, electrostatic forces and

Table 2
GP values of Laurdan emission calculated from fluorescent spectroscopy.

		Control	AS	CL	LS	DS1	DP1
DOPC	1 h	-0.094	0.004	-0.108	-0.092	0.036	0.019
	24 h	-0.104	0.061	-0.077	0.058	0.037	0.057
GUV ⁺	1 h	-0.124	0.179	-0.179	0.149	0.068	0.063
	24 h	-0.122	0.166	-0.133	0.179	0.200	0.149
GUV ⁻	1 h	-0.059	0.018	-0.009	-0.028	0.032	0.019
	24 h	-0.065	0.160	0.022	0.059	0.102	0.052

hydrogen bonds induce the same trend of phase change (gelation). Long exposure time increases the extent of membrane gelation (Table 2), because hydrogen bonds form gradually. Addition to the electrostatic forces and hydrogen bonds, hydrophobic effects were suggested to be taken into account for the stiffened membrane in a study on Au NPs that are different to SiO₂ NPs [34].

Fluid-phase membrane is necessary to support proteins and regulate molecular transport into and out of the cell [2,35]. Rigidified lipid membrane may disturb membrane signaling and trafficking, including lipid mediated endocytosis [34,36]. Therefore change in membrane fluidity is a possible reason of cytotoxicity because many important physiological activities require fluid cell membranes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhazmat.2015.01.063>.

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